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Differential Roles of the ChiB Chitinase in Autolysis and Cell Death of *Aspergillus nidulans*▽

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Autolysis is a natural event that occurs in most filamentous fungi. Such self-degradation of fungal cells becomes a predominant phenomenon in the absence of the regulator of G protein signaling FlbA in *Aspergillus nidulans*. Among a number of potential hydrolytic enzymes in the *A. nidulans* genome, the secreted endochitinase ChiB was shown to play a major role in autolysis. In this report, we investigate the roles of ChiB in fungal autolysis and cell death processes through genetic, biochemical, and cellular analyses using a set of critical mutants. Determination of mycelial mass revealed that, while the *flbA* deletion (Δ*flbA*) mutant autolyzed completely after a 3-day incubation, the Δ*flbA* Δ*chiB* double mutant escaped from hyphal disintegration. These results indicate that ChiB is necessary for the Δ*flbA*-induced autolysis. However, importantly, both Δ*flbA* and Δ*flbA* Δ*chiB* strains displayed dramatically reduced cell viability compared to the wild type. These imply that ChiB is dispensable for cell death and that autolysis and cell death are separate processes. Liquid chromatography-tandem mass spectrometry analyses of the proteins that accumulate at high levels in the Δ*flbA* and Δ*flbA* Δ*chiB* mutants identify chitinase (ChiB), dipeptidyl peptidase V (DppV), *O*-glycosyl compound hydrolase, β-*N*-acetylhexosaminidase (NagA), and *myo*-inositol-1-phosphate synthase (InoB). Functional characterization of these four genes reveals that the deletion of *nagA* results in reduced cell death. A working model bridging G protein signaling and players in autolysis/cell death is proposed.

Autolysis can be described as enzymatic self-degradation of the cells. It involves the activation of several key enzyme classes, resulting in the catabolism of macromolecules within the cell (11, 12, 23). Autolysis is observed in most filamentous fungi at the late stages of cultures and is affected by aging, programmed cell death, development, nutrient limitation, and numerous other factors (39). Despite its fundamental importance in growth, differentiation, secondary metabolism, and heterologous protein production, autolysis is a poorly understood feature of fungal biology (26, 39). It is anticipated that the elucidation of the molecular mechanisms governing fungal autolysis would have great impacts on both fundamental and applied aspects of filamentous fungi.

The *Aspergillus nidulans* Δ*flbA* mutant exhibits autolysis as a predominant phenotype (18). FlbA is a regulator of G protein signaling (RGS) that negatively controls vegetative growth signaling, primarily mediated by a heterotrimeric G protein composed of FadA (Gα) and SfaD::GpgA (Gβγ) (31, 33, 34, 42, 43). Loss of *flbA* function causes prolonged activation of G protein signaling, which results in uncontrolled proliferation of hyphal mass, the blockage of sporulation, and hyphal disintegration (autolysis). Because nearly the entire mycelium disappears during autolysis of Δ*flbA* mutant strains, some component of this phenomenon is hypothesized to involve enzymatic degradation as observed during autolysis of aging fungal cultures (11, 12, 23).

In *A. nidulans*, the last step of autolysis is thought to be the degradation of the cell wall of empty hyphae, which is associated with increased proteinase and chitinase activities (10). There are 15 potential chitinase open reading frames (ORFs) in the genome of *A. nidulans*. Among these, the class V endochitinase ChiB was shown to play an important role in autolysis. The deletion of *chiB* considerably reduced the intracellular and extracellular chitinase activities, and the levels of ChiB significantly increased when the fungal cells were starved for carbon sources, an induced condition for hyphal autolysis of *A. nidulans* (41).

In the present study we addressed two primary questions: (i) does ChiB function in the accelerated autolysis and/or cell death caused by loss of FlbA and (ii) are there other hydrolytic enzymes involved in autolysis and/or cell death in *A. nidulans*? In order to investigate a hypothetical connection between FlbA-controlled autolysis and the ChiB activity, we carried out genetic, biochemical, and cellular studies using the Δ*chiB*, Δ*flbA*, Δ*flbA* Δ*chiB* (double deletion), and *chiB* overexpression mutants. We found that, while ChiB plays a crucial role in Δ*flbA*-induced autolysis, ChiB is dispensable for cell death, corroborating the idea that cell death and autolysis are independent processes in *A. nidulans* (8). We further present the identification and partial functional characterization of four gene encoding the proteins accumulate at high level in Δ*flbA* and/or Δ*flbA* Δ*chiB* strains and propose a working model linking G protein signaling and autolysis.

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TABLE 1. *Aspergillus* strains used in this study

Strain	Genotype[a]	Source or reference
AF293	*A. fumigatus* WT	FGSC[b]
FGSC4	*A. nidulans* WT *veA*$^+$	FGSC
FGSC26	*biA1*	FGSC
A773	*pyrG89*; *wA3*; *pyroA4*	FGSC
PW1	*biA1*; *argB2*; *methG1*	P. Weglenski
RMS011	*pabaA1 yA2*; Δ*argB*::*trpC*$^+$; *trpC801*	37
RJYE07	*biA1* Δ*flbA*::*argB*$^+$; *methG1*; Δ*fadA*::*argB*$^+$ *trpC801*	13
RRAW16	*pyrG89 yA2*; *veA*$^+$	R. A. Wilson and N. P. Keller
TNJ12	*biA1*; *argB2* Δ*chiB*::*argB*$^+$; *methG1*	This study
TNJ13	*biA1*; *argB2*; *methG1*; *niiA*(p)::*chiB*::*argB*$^+$	This study
RNJ1.5	*pyrG89 yA2*; Δ*chiB*::*argB*$^+$	This study
RNJ1.8	*biA1* Δ*chiB*::*argB*$^+$	This study
RNJ3.1	*biA1* Δ*flbA*::*argB*$^+$	This study
RNJ3.5	*biA1* Δ*flbA*::*argB*$^+$; Δ*chiB*::*argB*$^+$	This study
TGS1.9	*pyrG89*; *wA3*; *pyroA4*; ΔAN2572.2::Af*pyrG*$^+$	This study
TGS2.2	*pyrG89*; *wA3*; *pyroA4*; ΔAN2395.2::Af*pyrG*$^+$	This study
TGS3.7	*pyrG89*; *wA3*; *pyroA4*; ΔAN1502.2::Af*pyrG*$^+$	This study
TGS4.2	*pyrG89*; *wA3*; *pyroA4*; ΔAN7625.2::Af*pyrG*$^+$	This study

[a] All strains carry the *veA1* mutation except for AF293, FGSC4, and RRAW16.
[b] FGSC, Fungal Genetics Stock Center (University of Missouri, Kansas City).

MATERIALS AND METHODS

Strains, media, and growth conditions. *A. nidulans* strains used in the present study are listed in Table 1. The fungal strains were grown on minimal solid or liquid medium with supplements (e.g., 25 μg of biotin/liter and 150 mg methionine/liter, referred to hereafter as MM) as described previously (15) and incubated at 37°C. For liquid submerged cultures, all strains were inoculated with 5×10^5 conidia/ml in 100 ml of liquid MM with 1% glucose (MMG) and incubated at 250 rpm at 37°C. Samples were taken at 1-day intervals for up to 8 days of cultivation.

Construction and growth of *A. nidulans* strains. The oligonucleotides used in the present study are listed in Table 2. To generate the *niiA*(p)::*chiB* fusion construct, the 1,197-bp *chiB* ORF derived from cDNA was PCR amplified using the primer pair of oNK-7 and oNK-8. The PCR product was then double digested with BamHI and EcoRI and cloned into pNQargB that contains the *A. nidulans niiA* promoter and the *Neurospora crassa qa-4* terminator (the present study). The resulting plasmid pNJ19 was then introduced into PW1. Multiple *chiB* overexpression strains were identified via PCR and Northern blot analyses. The *chiB* deletion mutant was generated by transforming PW1 with its deletion construct. The *chiB* deletion construct was generated by using the DJ-PCR method (44). The primer pairs oNK-1/oNK-3 and oNK-2/oNK-4 were used to amplify the 5′- and 3′-flanking regions of the *chiB* gene, respectively. The *argB* marker was amplified using the primer pair of oNK-9 and oNK-10. The fusion construct of the three amplicons was used as a template for the final PCR amplification of the *chiB* deletion construct using the primer pair of oNK-5 and oNK-6. Multiple Δ*chiB* transformants were isolated and verified as described previously (44). One Δ*chiB* transformant (TNJ12) was then meiotically crossed with RRAW16 to obtain RNJ1.5 and RNJ1.8 (Table 1). The Δ*flbA* Δ*chiB* double mutant (RNJ3.5) was isolated from the progeny of a meiotic cross between RNJ1.5 and RJYE07 (Table 1) and confirmed by a PCR-based method as described previously (44).

The deletion mutants of AN1502.2 (NagA), AN2395.2 (hydrolase), AN2572.2 (DppV), and AN7625.2 (InoB) were generated by using the DJ-PCR method (44). The 5′- and 3′-flanking regions of each gene were amplified using the primer pairs oNK-297/298 and oNK-299/300 (for NagA), oNK-303/304 and oNK-305/306 (for AN2395), oNK-309/310 and oNK-311/312 (for DppV), and oNK-315/316 and oNK-317/318 (for InoB), respectively, using FGSC4 genomic DNA as a template. The *A. fumigatus pyrG* marker was amplified from the AF293 genomic DNA with the primer pair oJH-83/oJH-86. The three fragments for each gene were fused, and each deletion cassette amplicon was further amplified with the nested primer pairs oNK-301/302 (for NagA), oNK-307/308 (for AN2395), oNK-313/314 (for DppV), and oNK-319/320 (for InoB), respectively. Each final amplicon was introduced into A773, and the deletion mutants were isolated and confirmed (44). For each gene, at least three deletion mutants were isolated.

Determination of mycelial mass and cell viability. The dry weight of the mycelium was determined as described previously (41). Fungal cell viability was determined by the percent reduction of Alamar Blue (AB) using a AlamarBlue cell viability assay kit (Biotium, Inc.) with a slight modification as follows. The AB assay reagent was placed into each test well of a 24-well plate (Nunc), which contained 2 ml of fresh medium and 1 ml of individual cultures with an equal amount of the mycelium, at a final concentration to 10% of the reaction volume. After the plate was incubated at 37°C for 6 h in the dark, the absorbance of each well was read at 570 and 600 nm. The percent reduction of AB was calculated as described previously (2, 22). The results are expressed as the mean ± the standard deviation for triplicates of individual cultures.

Protein extraction and gel electrophoresis. The mycelium was suspended in the lysis buffer (50 mM Tris-HCl [pH 7.2], 150 mM NaCl, 1% Triton X-100, 1 mM EDTA) and homogenized by using a Mini Bead-Beater (BioSpec Products). The homogenate was centrifuged in a microcentrifuge for 10 min at 15,000 rpm at 4°C, and the supernatant was used for further analyses. Proteins in concentrated supernatants (~100 μg) were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) with a stacking gel using the method of Laemmli (17) in a 7.5% acrylamide separating gel. Gel was then stained using GelCode Blue Stain Reagent (Thermo Scientific) according to the manufacturer's instructions.

Nano-LC-ESI-MS/MS and data analyses. All tandem mass spectrometry (MS/MS) experiments for peptide identification were performed by using an LTQ-MS (Thermo Finnigan) equipped with a nano-electron spray ionization (nano-ESI) source. From each sample, 10 ml was loaded by using an autosampler (Surveyor) onto a C_{18} trap column (inner diameter, 300 mm; length, 5 mm; particle size, 5 mm; LC Packings) for desalting and concentration at a flow rate of 20 ml/min. The trapped peptides were then back-flushed and separated on a homemade microcapillary column (150 mm in length) packed with C_{18} (particle size, 5 mm) in 75-mm silica tubing (8 mm inner diameter orifice). The mobile phases A and B were composed of 0 and 80% acetonitrile, respectively, each containing 0.1% formic acid. The gradient began at 5% phase B for 15 min, was ramped to 20% phase B for 3 min, to 60% for 45 min, to 95% for 2 min and, finally, held at 95% phase B for 7 min. The column was equilibrated with 5% phase B for 10 min before the next run. MS and MS/MS spectra were obtained at a heated capillary temperature of 220°C, an ESI voltage of 2.5 kV, and a collision energy setting of 35%. Data-dependent peak selection of the nine most abundant MS ions from MS was used. The previously fragmented ions were excluded for 60 s. The proteins were identified by searching fungi subset (219,981 entries) of the National Center for Biotechnology Information protein databases using the MASCOT 2.0 search algorithm (Matrix Science). The general parameters for search were considered to allow a maximum of one missed cleavage, 10 ppm of peptide mass tolerance, and the modifications of N-terminal Gln to pyro-Glu, oxidation of methionine, acetylation of protein N terminus, carbamidomethylation of cysteine, and acrylamide modified cysteine. A peptide charge state of 2 or 3, and fragment mass tolerance of 0.5 Da were used for the MS/MS ion search.

RESULTS

ChiB is in part required for Δ*flbA* induced autolysis in air-exposed culture. A potential role of *chiB* in Δ*flbA*-induced autolysis was investigated by examining wild type (WT), *chiB* overexpression (OE*chiB*), Δ*flbA*, Δ*chiB*, and Δ*flbA* Δ*chiB* (double-deletion) mutant strains. We reasoned that if ChiB was necessary for accelerated autolysis caused by Δ*flbA*, the deletion of *chiB* would result in (at least partial) suppression of the autolytic phenotype and may even restore asexual spore formation in the Δ*flbA* mutant. When point-inoculated on solid medium, whereas the WT, OE*chiB*, and Δ*chiB* colonies produced a lawn of the spore-bearing structures conidiophores (Fig. 1, see the center and edge), both Δ*flbA* and Δ*flbA* Δ*chiB* mutant colonies continued to accumulate undifferentiated hy-

TABLE 2. Primers used in this study

Primer	Sequence (5′–3′)[a]	Position or purpose
oNK-1	TCCAGTAAATAGGTAGATAG	5′ flanking region of *chiB*
oNK-2	ACAGCACTGTTCAACACGAC	3′ flanking region of *chiB*
oNK-3	*AGTCAAATGAGGCCTCTAAACTG*GTCACGTGGTTGCGGTTAAGTGC	3′ *chiB* with the An*argB* tail
oNK-4	*AGCCAAGGTAGATCCAGGCCTAA*CACAGGTAAGGTTAGGGTCGAGT	5′ *chiB* with the An*argB* tail
oNK-5	CATCCACATTCCCATCATGT	5′ nested for *chiB*
oNK-6	CGTTTGGTGTTCAGCGCACA	3′ nested for *chiB*
oNK-7	ATAT**GGATCC**ATGTCTGGATACAAAACTGTTG	5′ *chiB* with BamHI
oNK-8	ATAT**GAATTC**CTAGGACGACGGGAAGCCAG	3′ *chiB* with EcoRI
oNK-9	TGACCAGTTTAGAGGCCTCATTTGACT	5′ An*argB*
oNK-10	TGTGTTAGGCCTGGATCTACCTTGGCT	3′ An*argB*
oJH-83	GGATGTATCGTGACTGGCCTTCGG	5′ Af*pyrG*
oJH-86	TAATTCGCGGCATACGGTGTCTAA	3′ Af*pyrG*
oNK-297	TCAAGGAATGACTCGATCTCCCAG	5′ flanking region of AN1502.2
oNK-298	*GCTTTGGCCTGTATCATGACTTCA*ATAGGAAGTTGTAGGATTGATTC	3′ AN1502.2 with the Af*pyrG* tail
oNK-299	*ATCGACCGAACCTAGGTAGGGTA*TGACCAGACAGTCCTTCACTAGTG	5′ AN1502.2 with the Af*pyrG* tail
oNK-300	ACGAGCAGATAAGGTGGACCCATC	3′ flanking region of AN1502.2
oNK-301	TCATCTTGCATGAAGCTGTGTCG	5′ nested for AN1502.2
oNK-302	ACTTGAGATATGCATAATGCGTTG	3′ nested for AN1502.2
oNK-303	ATCATAACGCATGACCTGACCAC	5′ flanking region of AN2395.2
oNK-304	*GCTTTGGCCTGTATCATGACTTCA*TTCCTTCTACTTGTAACTTCCACC	3′ AN2395.2 with the Af*pyrG* tail
oNK-305	*ATCGACCGAACCTAGGTAGGGTA* GCTGATGGGCACCGGAATACATG	5′ AN2395.2 with the Af*pyrG* tail
oNK-306	TGGAATTGGTGAGCAACAGAGCTA	3′ flanking region of AN2395.2
oNK-307	GTGGCCGAGATTAAGCCCTGTGG	5′ nested for AN2395.2
oNK-308	TCTAGCCGTGGAGTCTTAGATTC	3′ nested for AN2395.2
oNK-309	ACTGACGCAGAAGCTGACTCTTG	5′ flanking region of AN2572.2
oNK-310	*GCTTTGGCCTGTATCATGACTTCA*GAGAGCACCCATACTGAAGCTGA	3′ AN2572.2 with the Af*pyrG* tail
oNK-311	*ATCGACCGAACCTAGGTAGGGTA*CTGCTTAAGGAGGGTATGTATAGT	5′ AN2572.2 with the Af*pyrG* tail
oNK-312	GCATCACTGATAGGAAATTATACTC	3′ flanking region of AN2572.2
oNK-313	AGACATGAACAACGAACGTCCTG	5′ nested for AN2572.2
oNK-314	CATAGCATTCGGTATAAGAGTGC	3′ nested for AN2572.2
oNK-315	TCCAGACTGTGGAGTGCTAGACG	5′ flanking region of AN7625.2
oNK-316	*GCTTTGGCCTGTATCATGACTTCA* AGCCATTGTGTCAGAATCTCAGC	3′ AN7625.2 with the Af*pyrG* tail
oNK-317	*ATCGACCGAACCTAGGTAGGGTA*ACTCTTGAGCACAAGCTGTTCTAG	3′ AN7625.2 with the Af*pyrG* tail
oNK-318	TCTGTAGTCTTTAGCCACGTCCTG	3′ flanking region of AN7625.2
oNK-319	TGGAGTAACCCACGATATGCTAG	5′ nested for AN7625.2
oNK-320	GTCTTTAGCCACGTCCTGGAGAG	3′ nested for AN7625.2

[a] Tail sequences are indicated in italics. Restriction sites are indicated in boldface.

phal mass, indicating that the deletion of *chiB* does not suppress the developmental defects caused by Δ*flbA*. However, careful examination of the Δ*flbA* and Δ*flbA* Δ*chiB* colonies revealed that the center of the colonies showed clearly different degrees of hyphal disintegration. Whereas Δ*flbA* strain exhibited nearly complete autolysis of the colony (the autolytic zone indicated by the arrow), the Δ*flbA* Δ*chiB* mutant showed partial autolysis, where the center region was opaque (Fig. 1, bottom), and this partially autolyzed region is restricted (see the arrow in Fig. 1). The close-up view of the center of Δ*flbA* Δ*chiB* mutant showed the remaining of the mycelia escaped from autolysis (Fig. 1, center white arrow). These results indicate that ChiB is in part necessary for Δ*flbA*-induced autolysis *A. nidulans* in air-exposed culture.

ChiB is necessary but not sufficient for autolysis in submerged culture. We further investigated a potential role of ChiB in Δ*flbA*-induced autolysis in submerged culture. The conidia of WT, OE*chiB*, Δ*flbA*, Δ*chiB*, and Δ*flbA* Δ*chiB* strains were inoculated into liquid MMG, and the morphologies and dry weights of the mycelia were examined. As shown in Fig. 2, the distinct phenotypes of both the Δ*flbA* and Δ*flbA* Δ*chiB* cultures were the lack of brown pigment accumulation and the loosened mycelial pellets. It is important to note that, whereas the mycelial pellets of both Δ*flbA* and Δ*flbA* Δ*chiB* strains were not as compact as those of the other three strains at the first day of incubation, clear differences in the morphologies of the mycelial pellets of Δ*flbA* and Δ*flbA* Δ*chiB* strains were detectable from day 3, when cellular autolysis began to proceed (Fig. 2). The Δ*flbA* hyphae started to disintegrate at day 3 and underwent complete autolysis at 4 days of incubation (see the arrow in Fig. 2). However, the Δ*flbA* Δ*chiB* mycelial pellets retained the abnormal cottonlike morphology without any sign of autolysis even at 8 days of incubation (for reference, see the photomicrograph of the 3-day-old mycelial pellets in Fig. 2). Moreover, mycelial dry weight of Δ*flbA* Δ*chiB* strain remained unchanged after day 4. These results indicate that ChiB is necessary for the Δ*flbA* induced autolysis in submerged culture. Consistent with this observation, we found that, while the WT and OE*chiB* strains showed similar patterns of morphological and growth changes (a sharp reduction of hyphal weight after 3 days), the Δ*chiB* strain exhibited a significantly delayed autolysis (Fig. 2). However, since overexpression of *chiB* did not accelerate or enhance hyphal disintegration, ChiB is necessary but not sufficient for autolysis in submerge-cultured *A. nidulans*.

ChiB is dispensable for cell death. A previous study presented evidence that apoptotic cell death and autolysis are separate biological processes regulated independently (8). We

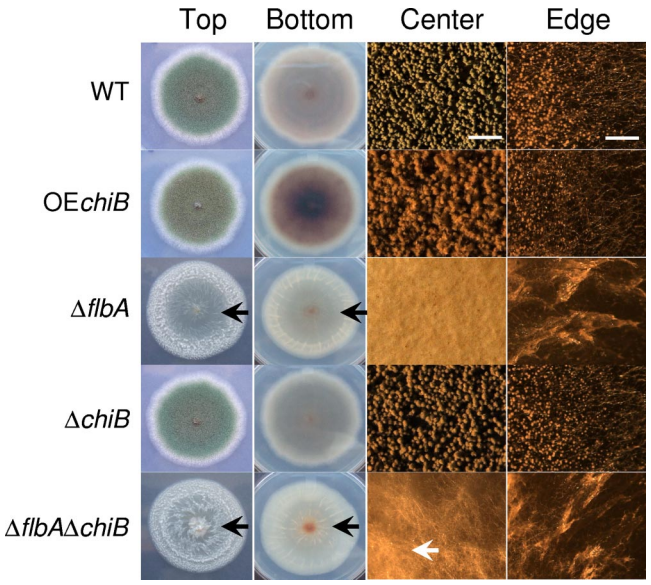

FIG. 1. Phenotypes of WT (FGSC26), OE*chiB* (TNJ13), Δ*flbA* (RNJ3.1), Δ*chiB* (RNJ1.8), and Δ*flbA* Δ*chiB* (RNJ3.5) strains on solid medium. The conidia of each strain were point inoculated in the center of solid MMG and incubated at 37°C for 3 days. Photographs of the top and bottom views of the entire colonies and close-up views of the center and edge of individual colonies (bar, 200 μm) are shown. Note that the Δ*flbA* Δ*chiB* mutant colony exhibits reduced levels of hyphal disintegration compared to the Δ*flbA* colony. The centers of the two colonies (marked by black and white arrows) show a clear difference in the remaining of undifferentiated hyphae and the autolytic zones.

checked whether the absence of *chiB* would also influence cell death by determining the percent AB reduction, which corresponds to the activity of the living cell mitochondria (Fig. 3). All strains tested maintained equally high levels of cell viability until day 2, and then they began to show evident differences at day 3. Importantly, unlike autolysis, both Δ*flbA* and Δ*flbA* Δ*chiB* strains showed a rapid reduction of cell viability at day 3 and <10% of AB reduction at day 4, indicating that a majority of the cells were nonviable. Furthermore, both WT and Δ*chiB* strains displayed similar cell death pattern from day 1 to day 8. These indicate that ChiB is not required for cell death in *A. nidulans* and corroborate the idea that apoptotic cell death and autolysis are separate biological processes. However, since the overexpression of *chiB* caused a clear acceleration of cell death (Fig. 3), a potential role of ChiB in cell death cannot be excluded fully.

Identification of five proteins upregulated in Δ*flbA* and/or Δ*flbA* Δ*chiB* strains. In an attempt to identify proteins present at high levels during autolysis and/or cell death, we looked for the proteins upregulated in Δ*flbA* and/or Δ*flbA* Δ*chiB* strains. The total intracellular proteins of WT, Δ*flbA*, and Δ*flbA* Δ*chiB* strains grown 2 days in liquid submerged culture were analyzed by using SDS-PAGE, followed by nano-liquid chromatography (LC)-ESI-MS/MS. Five distinct proteins (I to V) accumulated at high levels in Δ*flbA* and/or Δ*flbA* Δ*chiB* strains with relative molecular masses approximately 82, 71, 68, 54, and 45 kDa, respectively (Fig. 4). These five protein bands were excised and subjected to in-gel trypsin digestion, followed by LC-MS/MS analyses. The MASCOT scores of each band were significant levels (922, 1225, 644, 525, and 10928, respectively). Band V (not present in the Δ*flbA* Δ*chiB* strain) was identified as ChiB, with high sequence coverage (53%). Bands I, II, and III were identified as probable dipeptidyl peptidases similar to DppV in *Aspergillus fumigatus* (3) and thus named DppV, hydrolase for *O*-glycosyl compounds (AN2395.2), and β-*N*-acetylhexosaminidase (NagA [16]), respectively (Table 3). Most of the identified proteins are predicted hydrolytic enzymes, except for band IV *myo*-inositol-1-phosphate synthase (MIPS; InoB in *A. nidulans*; ABX82889), which is predicted to catalyze the first step of the de novo synthesis of *myo*-inositol phosphate. The peptides of bands I to V showing significant matches are shown in Table 4. The scores of individual ions are defined by the absolute probability that the observed match is a random event. Scores of individual ions higher than 19 indicate significant identity or extensive homology ($P < 0.05$).

NagA may be associated with cell death. To begin to dissect the potential roles of these four proteins in cell death and/or autolysis, we generated multiple (two to four) deletion strains of each gene using FGSC A773 as a recipient strain. No deletion mutants displayed distinct abnormalities in hyphal growth and/or development on solid medium. However, when tested for altered mycelial morphologies or cell death rates in liquid submerged culture using the AB reduction, these deletion mutant strains displayed a clear difference. As shown in Fig. 5, while the reduction rates of all tested strains, including the WT control (A773), were greater than 92% at day 1, the reduction rate of the Δ*nagA* mutant was significantly higher (85%) than that of WT (62%), Δ*dppV* (61%), ΔAN2395.2 (47%), and Δ*inoB* (36%) strains at day 2, indicating that NagA may be involved in the control of cell death. Perhaps somewhat surprisingly, the absence of InoB or AN2395.2 (a probable hydrolase) appeared to cause accelerated cell death and disorganization of mycelial pellets (Fig. 5), suggesting that these proteins might be needed for the controlled progression of cell death. In these experimental sets, we consistently observed that all strains reached nearly complete cell death at day 3 (in comparison to day 8 in Fig. 3). Although the exact reason for such a difference is not known, it is important to note that FGSC A773 carries the *wA3* mutant allele, resulting in the colony with white conidia and potentially weekend cell walls. These characteristics may have contributed to a difference in the timing and progression of cell death between A773 and FGSC A26 (see Fig. 3). The effects of the deletion of these genes on autolysis and their potential involvement in FadA-mediated signaling remain to be investigated.

DISCUSSION

The fungal cell wall is composed of chitin, glucans, and other biopolymers that are cross-linked with each other (1). Autolysis of filamentous fungi is a natural process of self-disintegration of aged fungal cells and cell walls, resulting from the activities of a diverse array of hydrolytic enzymes such as proteases, glucanases, and chitinases (39). The present study focuses on revealing a genetic link between upstream heterotrimeric G protein signaling and the ChiB chitinase and the identification of the proteins that are upregulated during autolysis and/or cell death in the model filamentous fungus *A. nidulans*.

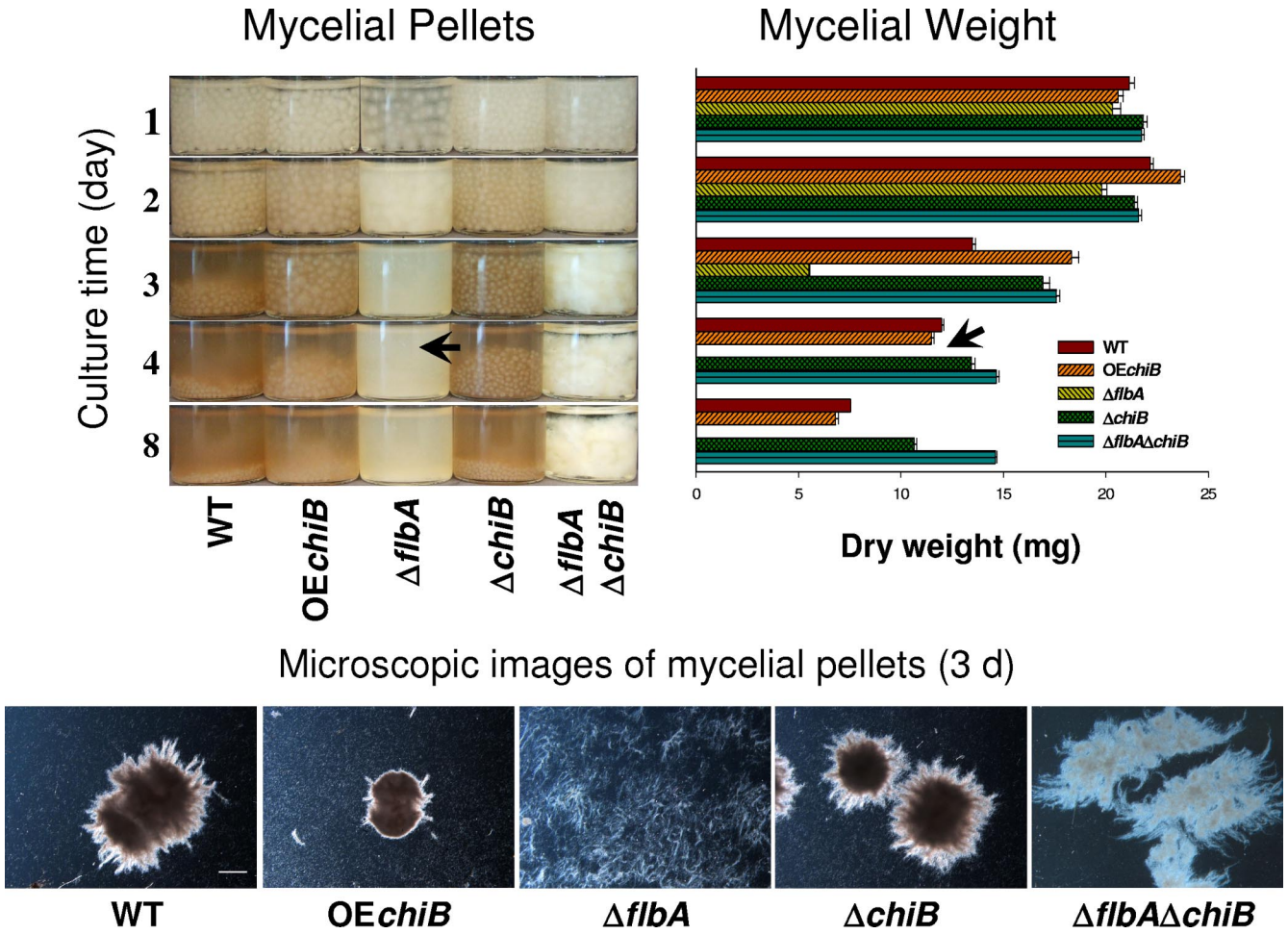

FIG. 2. Phenotypes and mycelial dry weights of WT (FGSC26), OE*chiB* (TNJ13), Δ*flbA* (RNJ3.1), Δ*chiB* (RNJ1.8), and Δ*flbA* Δ*chiB* (RNJ3.5) strains in liquid MMG submerged culture. The mycelium of Δ*flbA* strain (RNJ3.1) autolyzed completely upon 4 days of incubation, whereas Δ*flbA* Δ*chiB* strain (RNJ3.5) maintained abnormal cottonlike mycelial pellets even at day 8. The photomicrographs of the mycelial pellets of individual strains (bar, 1.0 mm) were taken at day 3. Note that, while Δ*flbA* strain shows fragmented mycelial pellets due to accelerated autolysis, Δ*flbA* Δ*chiB* strain exhibits nonautolyzed cottonlike mycelial pellets.

FlbA is an RGS that negatively controls G protein-mediated proliferation signaling likely by acting as a GTPase activating protein for FadA Gα (43) (Fig. 6). The absence of FlbA function causes constitutive activation of vegetative growth, which results in the "fluffy-autolytic" phenotype (42). Our studies demonstrate that ChiB may play a specific role in autolytic signaling (Fig. 1 and 2) in that Δ*flbA* Δ*chiB* strain formed nonautolytic cottonlike mycelial pellets in liquid submerged culture (Fig. 2). Moreover, there were no considerable differences among the phenotypes of WT, OE*chiB*, and Δ*chiB* strains in autolysis. These findings suggest that ChiB plays a differential role in autolysis and that other hydrolytic enzymes may be involved in the autolysis of *A. nidulans*. This is in agreement with a previous report that found numerous hydrolytic enzymes, including chitinase, laminarinase, protease, and *N*-acetylglucosaminidase, to be associated with the autolytic process in carbon-starved *A. nidulans* cultures (1, 27).

Although ChiB plays an important role in autolysis likely via cell wall chitin degradation, it is found to be dispensable for cell death. In contrast to the dry mass differences, the cell viability of the Δ*flbA* and Δ*flbA* Δ*chiB* mutants dramatically decreased in a similar manner (Fig. 3). These findings support the idea that autolysis and cell death are separately regulated processes. Are other chitinases associated with cell death? The *A. nidulans* genome has 15 ORFs predicted to contain conserved domains for the active site of glycosyl hydrolase family 18 (28). Only a few of these ORFs have been functionally characterized, and these include *chiA* (38, 40) and *chiB* (41). ChiA is a glycosylphosphatidylinositol-anchored chitinase that functions in cell wall remodeling and/or cell wall maturation (40). Another chitinase, a 27-kDa endochitinase purified from the autolytic cultures of *A. nidulans*, was shown to have the ability to hydrolyze the cell wall chitin (29). In addition, NagA (*N*-acetyl-β-D-glucosaminidase [9]) was shown to play a role in morphogenesis and nutrient utilization during autolysis (28). Our study of the identification and partial characterization of NagA revealed that it plays a potentially important role in cell death (see below).

In addition to ChiB, four additional proteins accumulate at high levels in Δ*flbA* and/or Δ*flbA* Δ*chiB* strains (Fig. 4). Protein

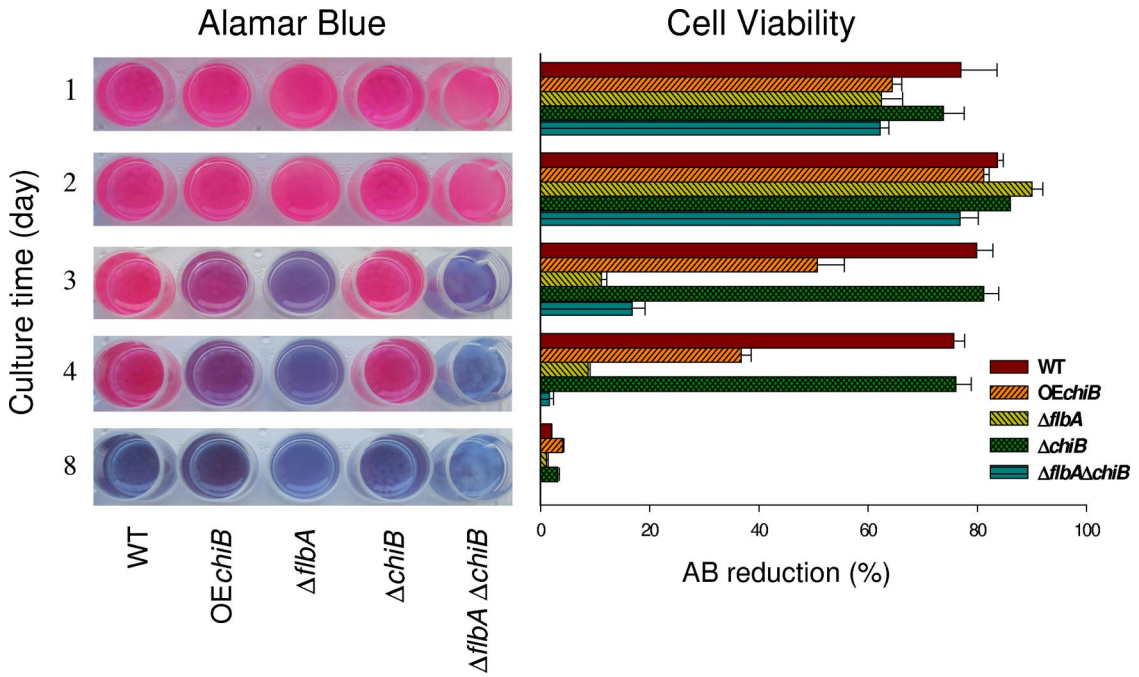

FIG. 3. AB reduction analyses of five *A. nidulans* strains. Note that the color of Δ*flbA* and Δ*flbA* Δ*chiB* strains began to turn into blue as early as day 3 of incubation.

I (AN2572.2; 719 amino acids) shows extremely high homology (75% identity, E-value = 0) with the *A. fumigatus* dipeptidyl peptidase DppV (721 amino acids [3]). It can be speculated that during *Aspergillus* autolysis (or cell death), the extracellular matrix of the fungus is degraded and the products of hydrolysis of the proteases could then be processed by the secreted type V dipeptidyl peptidase. The metabolically active fungal cells could then use these dipeptides as a source for amino acids for the cellular growth or maintenance. The band II AN2395.2 (613 amino acids) belongs to the family of *O*-glycosyl hydrolases (EC 3.2.1) that are an extensive group of enzymes that hydrolyze the glycosidic bond between two or more carbohydrates or between a carbohydrate and a noncarbohydrate moiety (InterPro). Particularly, AN2395.2 is predicted to have β-galactosidase, β-mannosidase, and β-glucuronidase activities and thus to degrade various sugar polymers in the cell wall. Band III is turned out to be NagA (β-*N*-acetylglucosaminidase; GlcNAcase [16]). Enzymatic degradation of chitin in microorganisms occurs in two consecutive steps (4). First, chitin (β-1,4-linked polymer of *N*-acetylglucosamine) is broken down into multiunit *N*-acetylglucosamine $(GlcNAc)_n$ molecules by exo (EC 3.2.1.29)- and endo (EC 3.2.1.14)-chitinase. Second, the resulting $(GlcNAc)_n$ is then hydrolyzed into GlcNAc by β-*N*-acetylglucosaminidase (4, 16). Thus, we can postulate that ChiB (band V) and NagA accumulate at high levels during *Aspergillus* autolysis and/or cell death and thereby coordinately confer cell death and degradation of fungal cell walls. In fact, Pusztahelyi et al. (28) demonstrated that the levels of *nagA* and *chiB* mRNA were high during the stationary and autolytic phases.

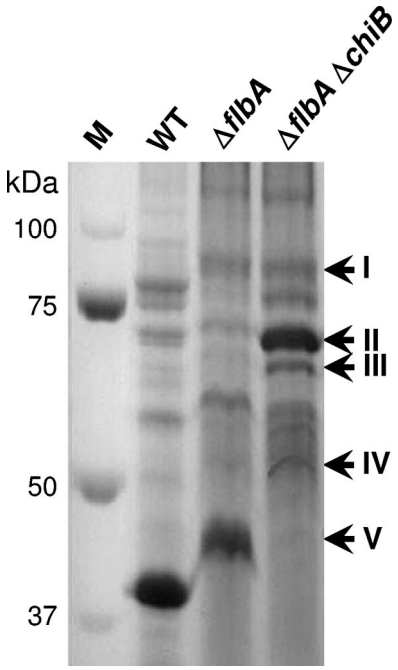

FIG. 4. SDS-PAGE images of intracellular proteins isolated from WT, Δ*flbA*, and Δ*flbA* Δ*chiB* strains grown in liquid MMG. Protein portions of ca. 100 μg were loaded onto each lane.

While it is somewhat straightforward to devise the potential functions of the above-mentioned hydrolytic enzymes in autolysis, a role for MIPS (InoB in *A. nidulans*; AN7625.2; 534 amino acids) in autolysis is not so obvious. MIPS (EC 5.5.1.4) catalyzes the conversion of D-glucose-6-phosphate to 1L-*myo*-inositol-1-phosphate, which is the first committed and rate-limiting step in the production of all inositol-containing com-

TABLE 3. Proteins induced in the Δ*flbA* and/or Δ*flbA* Δ*chiB* mutants

Band	Annotation	ORF (locus)	EMBL no.	MASCOT score	Sequence coverage (%)	M_r		Biological process and potential molecular function
						Actual (gel)	Predicted	
I	Uncharacterized protein	AN2572.2	EAA64677.1	922	28	81,600	79,397	Proteolysis DppV, dipeptidyl peptidase V
II	Uncharacterized protein	AN2395.2	EAA64506.1	1225	27	70,600	68,464	Carbohydrate metabolism hydrolase for *O*-glycosyl compounds
III	*N*-Acetylglucosaminidase	AN1502.2 (*nagA*)	BAB13330.1	644	24	67,900	67,941	Carbohydrate metabolism NagA, β-*N*-acetylhexosaminidase
IV	Uncharacterized protein	AN7625.2 (*inoB*)	EAA61811.1	525	31	53,500	51,633	Phospholipid biosynthesis InoB, inositol-3-phosphate synthase
V	Chitinase	AN4871.2 (*chiB*)	BAA35140.2	10928	53	44,600	44,178	Chitin catabolism ChiB, chitinase

pounds (21). Inositol-containing compounds are crucial for various biological processes, including membrane trafficking, storage of molecules, signal transduction, second messenger signaling, cell wall biogenesis, and the production of stress-related molecules (7, 20, 24, 36). In addition, *myo*-inositol polyphosphates take part in chromatin remodeling, regulation of gene expression, and mRNA export (25, 35). Furthermore, the *Saccharomyces cerevisiae INO1* gene encoding a MIPS is regulated at the level of transcription by the soluble precursor inositol and choline (5, 14). Collectively, one can postulate that the upregulated production of InoB might be associated with triggering signaling governing autolysis, cell death, or cell wall

TABLE 4. Peptides identified by LC-ESI-MS/MS analyses of five protein bands

Band	Amino acid positions	Peptide sequence	Mol wt		Ion score	Rank
			Expected	Calculated		
I	169–176	IYDSIYVR	1,027.5	1,027.5	66	1
	210–238	NLVAPIPNAESPYPPFGGSSDYDISADGK	2,978.4	2,977.3	76	1
	315–324	VIYIYDLASK	1,183.6	1,183.6	68	1
	346–366	STIVAGSEDEGSGNLFLIPVK	2,133.1	2,132.1	110	1
	578–591	ALVTHDGTFVADAK	1,443.7	1,443.7	70	1
	592–602	ISTEELWFM*ER	1,455.6	1,455.6	71	1
	644–659	LPVAEGLSLFNVLQER	1,785.0	1,783.9	79	1
II	172–195	GSNELLVFVYDPTDTSGTMVPLGK	2,540.2	2,539.2	68	1
	254–275	VTIEDGSDTVATHSGSANTEFK	2,266.0	2,265.0	108	1
	331–351	FLFGTLDQGFWPDGLYTPPTR	2,428.2	2,427.1	76	1
	402–413	TPNAAQQVEFAR	1,330.6	1,330.6	88	1
	414–423	QLELLINQLK	1,210.7	1,210.7	80	1
	549–562	AHYLLSELTEQVTK	1,630.8	1,630.8	98	1
III	137–147	AGSDAIDITAK	1,060.5	1,060.5	89	1
	203–215	KLHEQLDGMALSK	1,468.7	1,468.7	92	1
	569–584	EYLLANGVM*AATVVPK	1,691.9	1,690.8	95	1
IV	24–42	APLFTVNSPNVEYTETEIK	2,152.0	2,151.0	99	1
	116–130	AANYYGSLVMSSTIK	1,619.7	1,619.7	115	1
	190–213	EM*AEM*KPLPSVYYPDFIAANQEDR	2,846.3	2,845.2	78	1
	247–257	VIVMWTANTER	1,334.6	1,334.6	72	1
	258–277	YADILPGVNDTAENLVNSIK	2,146.1	2,145.0	100	1
	338–350	SALVDFLINAGIK	1,359.7	1,359.7	80	1
V	6–18	TVGYFVNWAIYGR	1,544.7	1,544.7	72	1
	91–114	VLLSIGGWTYSPNFTNGAGTPENR	2,551.2	2,550.2	71	1
	158–167	EALNAAQGQR	1,056.5	1,056.5	69	1
	168–184	RFQLTVAVPAGPDNYNK	1,889.9	1,888.9	74	1
	260–287	AFQNTDGPGRPYSGIGQGTWEQGV YDYK	3,091.4	3,090.4	71	1
	314–327	EMVSYDTVAAADLK	1,511.7	1,511.7	115	1
	336–348	LGGAM*WWETSADK	1,466.6	1,466.6	85	1
	356–376	ADGSLIGTFVEDVGGVNNLDR	2,148.0	2,147.0	116	1
	377–392	TQNAISYPDSQYDNLK	1,856.8	1,855.8	73	1

[a] M*, methionine oxidation.

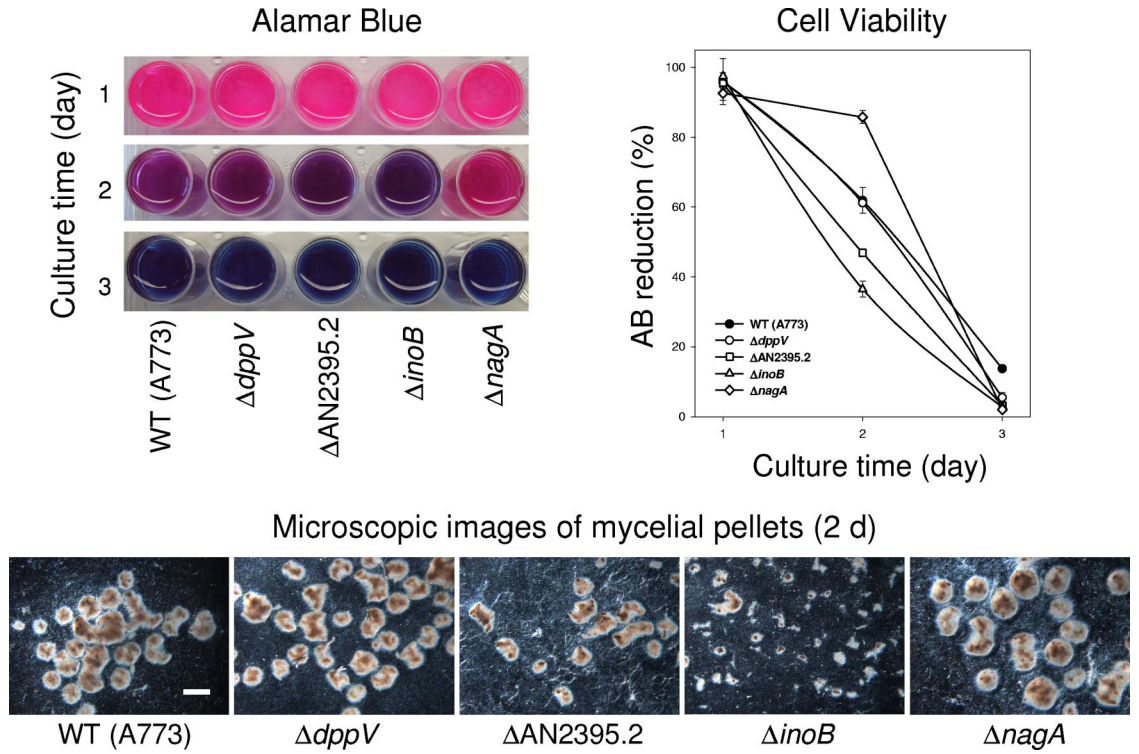

FIG. 5. Cell viability of WT, Δ*dppV*, ΔAN2395.2, Δ*inoB*, and Δ*nagA* strains determined by AB reduction rates. Note the color difference of the Δ*nagA* mutant cell extract at day 2. The mycelial pellets of the ΔAN2395.2 and Δ*inoB* strains were somewhat disintegrated and reduced in size at day 2 (bar, 1.0 mm).

biogenesis and/or complex transcriptional control of the *inoB* gene during autolysis in *A. nidulans*.

The postulated biological roles of four proteins were partially studied by phenotypic analyses of the corresponding deletion mutants. Examination of cell viability of the four mutants led to the finding that the β-*N*-acetylglucosaminidase NagA might be involved in cell death (Fig. 5). The fact that NagA is present at high levels in the Δ*flbA* Δ*chiB* strain (but not in the Δ*flbA* strain) (Fig. 4) suggests that the absence of both FlbA and ChiB functions may cause enhanced accumulation of NagA (and other hydrolytic enzymes). *A. nidulans* produces more than two types of β-*N*-acetylglucosaminidases during autolysis. These are thought to be involved in various biological processes requiring cell wall digestion, such as the germination of conidia, tip growth of hyphae, and hyphal autolysis (16, 30). A previous study partially characterized NagA and reported that no clear phenotypic differences between WT and Δ*nagA* strains were observable (16). Kim et al. concluded that NagA was not essential for the growth of the filamentous fungi on the medium containing an easily metabolized carbon source (16). Our study is the first report that defines a potential function of NagA in the process of cell death. Further investigation of a potential requirement for NagA (and other proteins) in Δ*flbA*-induced accelerated cell death and/or autolysis is in progress.

Finally, a genetic model linking heterotrimeric G protein signaling and ChiB and NagA is presented (Fig. 6). Previous studies showed that the chitin content of the cell wall of $fadA^{G203R}$ (a dominant interfering mutant allele [43]) and Δ*sfaD* (31) strains was about four times higher than that of the WT (6). These observations led Coca et al. (6) to suggest that the FadA- and SfaD-dependent signaling pathways negatively

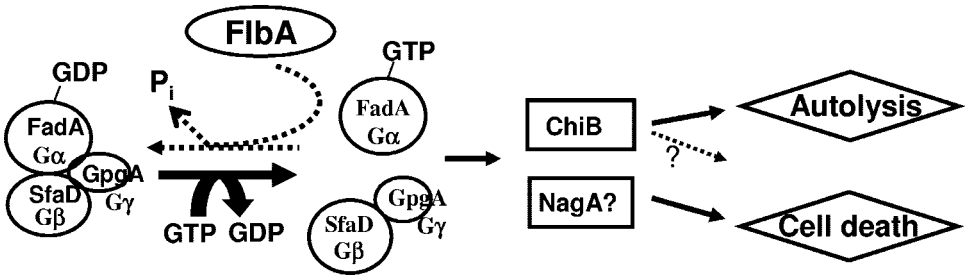

FIG. 6. Working model linking G protein signaling and autolysis and/or cell death in *A. nidulans*. ChiB and NagA may play differential roles in FadA-mediated autolysis and cell death signaling (see the text). A potential role of ChiB in cell death is indicated by a dotted arrow.

regulate the cell wall chitin content and cell wall structure in *A. nidulans*. Additional studies by Leiter et al. (19) showed that FadA-mediated signaling was involved in the initiation of apoptotic cell death in *A. nidulans*. In addition, the FadA heterotrimeric G protein complex was found to be a part of the signal transduction pathway that promotes apoptosis in hyphae exposed to the quorum-sensing molecule farnesol (32). Our results, in conjunction with these other findings, lead to a working model that constitutive activation of FadA (and SfaD) signaling by the absence of FlbA results in accelerated autolysis and cell death and that ChiB and NagA play differential roles in these interconnected, yet separate biological processes.

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